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S100A13 Participates in the Release of Fibroblast Growth Factor 1 in Response to Heat Shock *in Vitro**

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S100A13, a member of the S100 gene family of Ca^{2+} -binding proteins has been previously characterized as a component of a brain-derived heparin-binding multi-protein aggregate/complex containing fibroblast growth factor 1 (FGF1). We report that while expression of S100A13 in NIH 3T3 cells results in the constitutive release of S100A13 into the extracellular compartment at 37 °C, co-expression of S100A13 with FGF1 represses the constitutive release of S100A13 and enables NIH 3T3 cells to release S100A13 in response to temperature stress. S100A13 release in response to stress occurs with kinetics similar to that observed for the stress-induced release of FGF1, but S100A13 expression is able to reverse the sensitivity of FGF1 release to inhibitors of transcription and translation. The release of FGF1 and S100A13 in response to heat shock results in the solubility of FGF1 at 100% (w/v) ammonium sulfate saturation, and the expression of a S100A13 deletion mutant lacking its novel basic residue-rich domain acts as a dominant negative effector of FGF1 release *in vitro*. Surprisingly, the expression of S100A13 also results in the stress-induced release of a Cys-free FGF1 mutant, which is normally not released from NIH 3T3 cells in response to heat shock. These data suggest that S100A13 may be a component of the pathway for the release of the signal peptide-less polypeptide, FGF1, and may involve a role for S100A13 in the formation of a noncovalent FGF1 homodimer.

FGF1¹ and FGF2 are the prototype members of a large family of heparin-binding growth factor genes that regulate numerous biological processes such as neurogenesis, mesoderm formation, and angiogenesis (1, 2). FGF1 and FGF2 lack a classical signal peptide sequence that provides access to the conventional endoplasmic reticulum (ER)-Golgi secretion pathway, a characteristic that led to the hypothesis that the release of these polypeptides may proceed through novel release/export

pathways (2). Our laboratory previously demonstrated that FGF1, but not FGF2, is released as a latent homodimer by a transcription- and translation-dependent mechanism in response to a variety of cellular stresses including heat shock (3), hypoxia (4), and serum starvation (5). Conversely, the disruption of communication between the ER and Golgi apparatus by brefeldin A does not prevent the release of FGF1 from NIH 3T3 cells, confirming that FGF1 release may occur through a non-conventional pathway (6).

FGF1 is released *in vitro* as a reducing agent- and denaturant-sensitive complex, which contains the p40 extravesicular domain of the Ca^{2+} -binding protein, p65 synaptotagmin (Syt)1 (7). The release of FGF1 in response to stress is dependent on Syt1 expression, since the expression of either a deletion mutant lacking 95 amino acids from the extravesicular portion of Syt1 or an antisense-Syt1 gene is able to repress FGF1 release in NIH 3T3 cells (7, 8). In addition, FGF1 purified from ovine brain as a high molecular weight aggregate exists as a component of a noncovalent heparin-binding complex with p40 Syt1 and S100A13 (9).

The interleukin (IL)-1 gene family of proinflammatory polypeptides also contains functional cytokines that act as extracellular, receptor-dependent mediators of cellular function (10, 11), yet the majority of the members of the IL-1 gene family lack a classical signal peptide sequence for conventional secretion (10, 11). Interestingly, crystallographic analysis of the FGF and IL-1 prototypes demonstrates a remarkable level of structural similarity between these seemingly disparate polypeptides (12, 13). Recent studies have suggested that the pathway utilized for the release of IL-1 α is quite similar yet distinct from the pathway utilized by FGF1 export, since both proteins are released in response to temperature stress with similar biochemical and pharmacologic properties (14). In contrast, however, the IL-1 α release pathway requires the proteolytic processing of the 31-kDa precursor form of IL-1 α to the 17-kDa mature form for IL-1 α release and does not appear to utilize Syt1 for export, since a dominant negative Syt1 mutant blocks FGF1 but not IL-1 α release (14). Although we have not been able to eliminate the function of other Syt gene family members in the stress-induced release of IL-1 α , the observation that the precursor form of IL-1 α is able to block the release of FGF1 in response to temperature stress suggests that the FGF1 and IL-1 α release pathways may be mechanistically linked (14).

Recently, we have reported that amlexanox, an anti-allergic drug that binds S100A13 (15), is able to inhibit the release of FGF1 and p40 Syt1 in response to temperature stress (9, 16). Because amlexanox also induces a Src-dependent and reversible disassembly of actin stress fibers (16), these data have suggested a role for the actin cytoskeleton in the regulation of FGF1 release (16).

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¹ The abbreviations used are: FGF, fibroblast growth factor; DTT, dithiothreitol; ER, endoplasmic reticulum; IL, interleukin; PAGE, polyacrylamide gel electrophoresis; Syt, synaptotagmin.

S100A13 is a member of a large gene family of Ca^{2+} -binding proteins characterized by the absence of a classical signal peptide sequence and the presence of two Ca^{2+} -binding EF-hand domains (17, 18). S100A13 is a novel member of the S100 gene family that encodes a protein containing a positively charged carboxyl-terminal domain that may be involved in specific protein interactions (19). While members of the S100 gene family, including S100A13 (20), are expressed in a variety of tissues and organs (17, 18), the expression of a few members of the S100 gene family have been implicated in the regulation of human pathology including neurodegenerative diseases, cardiomyopathies, cancer, and chronic inflammation (17, 18). Interestingly, members of the S100 gene family have been shown to be constitutively released into the extracellular compartment (17, 18, 21), where they have been characterized as leukocyte chemoattractants and regulators of macrophage activation (17, 18, 22).

Since S100A13 was purified from the ovine brain as a part of the multiprotein aggregate containing FGF1 and p40 Syt1, we sought to determine whether S100A13 is involved in the release of FGF1. We report that S100A13 expression facilitates the release of FGF1 into the extracellular compartment in response to temperature stress *in vitro* and that S100A13 expression is able to revert the dependence for both transcription and translation in the release of FGF1 in response to heat shock. We further describe the unanticipated observation that S100A13 expression is able to export a Cys-free FGF1 mutant, suggesting that S100A13 may be involved in the formation of the FGF1 homodimer, a prerequisite for export.

MATERIALS AND METHODS

Plasmid Constructs—The plasmid pT7T3-S100A13 containing the cDNA encoding murine S100A13 was a generous gift from Dr. Beat W. Schafer (University of Zurich, Switzerland), and all restriction enzymes were obtained from New England Biolabs. The S100A13 cDNA was obtained by digesting the pT7T3-S100A13 plasmid with *NotI* and *BstZ171*. The fragment was cloned into the pCS2-6-Myc tag vector digested with *EcoRI*, filled-in by the Klenow reaction (New England Biolabs Inc.), and digested with *NotI*. The Myc tag was ligated at the amino terminus of the S100A13 protein and consisted of a 13-amino acid epitope (MEQKLISEEDLNE) repeated six times and recognized by the 9E10 monoclonal anti-Myc antibody (Oncogene Research Products). For expression in mammalian cells, the Myc-S100A13 DNA was digested using *HindIII* and ligated into the pcDNA3.1-Hygro (+) vector (Invitrogen) also excised with *HindIII*. For expression in prokaryotic cells, S100A13 cDNA was digested from pT7T3-S100A13 using *HindIII* and *BstZ171*. The fragment was ligated into the prokaryotic expression vector, pGEX-KG, digested with *XbaI*, filled-in by the Klenow reaction, and further digested with *HindIII*. The pGEX-KG vector contains the sequence encoding for the glutathione *S*-transferase (GST) that was used to purify the recombinant S100A13. For the preparation of the S100A13Δ88–98 mutant, the plasmid pT7T3-S100A13 was digested with *BsaBI* and *PpuMI*, and the S100A13 fragment was purified by gel electrophoresis. Two oligonucleotides lacking the sequence encoding for the last 11 amino acids of S100A13 were ligated in the digested plasmid. The two oligonucleotides and their complementary chains were designed with a *BsaBI* site at the 5'-end and a *PpuMI* site at the 3'-end and with a complementary overlapping sequence in the middle. The sequences of the oligonucleotides used are as follows: 5'-GAATCAGG-ACTCAGAGCTGAGGTTTCAGTGAATACTGGAGACTGAT-3'; 5'-AGT-ATTCACTGAACCTCAGCTCTGAGCTCTGATTC-3'; 5'-TGGAGAGCT-GGCAAGGAAGTCTAAAGCTTGCTGTCACAGCAG-3'; 5'-GTCCTGC-TGGACGCAAGCTTTAGACTTCCTTGCCAGCTCTCCAATCAGTC-TCC-3'. The S100A13Δ88–98 construct was ligated into the pCS2-6Myc tag vector with the six-Myc tag repeat sequence fused at the amino terminus of the protein and the Myc-S100A13Δ88–98 sequence cloned into the pcDNA3.1-Hygro (+) expression vector as described above.

Cell Culture—Murine NIH 3T3 cells were obtained from the American Type Culture Collection (ATCC) and grown as previously described (3). NIH 3T3 cells, FGF1 NIH 3T3 (3), and Cys-free FGF1 NIH 3T3 cell transfectants (6) were cotransfected with the gene encoding murine Myc-S100A13 cloned into the pcDNA3.1-Hygro (+) vector or with the

insertless expression vector. FGF1 NIH 3T3 cells were also transfected with the same eukaryotic expression vector containing the DNA sequence encoding for the Myc-S100A13Δ88–98 mutant. All transfections were performed using a multicomponent lipid-based reagent (FuGENE 6; Roche), and stable clones of the NIH 3T3 cell cotransfectants were obtained, expanded, and grown in Dulbecco's modified Eagle's medium (Cellgro) supplemented with 10% (v/v) bovine calf serum (HyClone), $1\times$ antibiotic/antimycotic (Life Technologies, Inc.), 0.15 g/liter hygromycin B (Life Technologies), and 0.4 g/liter Geneticin (G418; Life Technologies) on human fibronectin-coated dishes ($10\ \mu\text{g}/\text{cm}^2$).

Temperature Stress, Processing of Conditioned Media, and Immunoblot Analysis—NIH 3T3 cell transfectants were grown to 70–80% confluency, and prior to temperature stress, the cells were washed with Dulbecco's modified Eagle's medium containing 5 units/ml heparin (Upjohn). The heat shock was performed as previously described (3, 7, 8) in Dulbecco's modified Eagle's medium containing 5 units/ml heparin for 110 min at 42°C . Control cultures were incubated at 37°C in Dulbecco's modified Eagle's medium containing 5 units/ml heparin. Two independent clones from each transfection have been evaluated with similar results. The effects of 2-deoxyglucose (Sigma), brefeldin A (Epicenter Technologies), amlexanox (Takeda), latrunculin (Molecular Probes, Inc., Eugene, OR), actinomycin D (Sigma), and cycloheximide (Sigma) on FGF1 and Myc-S100A13 release were evaluated as previously reported (3, 6, 8, 9, 16). Following heat shock, the conditioned media were collected, filtered, and treated with either 0.1% (w/v) DTT (Sigma) for 2 h at 37°C or fractionated with ammonium sulfate (Sigma) at 100% (w/v) saturation as previously described (3, 6). After ammonium sulfate fractionation, conditioned media were centrifuged at $10,000\times g$ for 50 min, and the pellets were resuspended in 20 ml of 50 mM Tris, pH 7.4, containing 10 mM EDTA (TEB). Pellets and supernatants were dialyzed against 50 mM Tris, pH 7.4, for 18 h and treated with 0.1% (w/v) DTT for 2 h at 37°C . DTT- and ammonium sulfate-treated conditioned media were adsorbed to a heparin-Sepharose CL-6B (1-ml) column (Amersham Pharmacia Biotech), equilibrated with TEB, and the adsorbed proteins were washed with TEB and eluted with 2.5 ml of TEB containing NaCl concentrations specified under "Results." Eluted proteins were concentrated using Centricon 10 (Amicon, Inc.), resolved by 15% (w/v) SDS-PAGE, transferred to a nitrocellulose membrane (Hybond C; Amersham Pharmacia Biotech), and immunoblotted with an anti-Myc monoclonal antibody (Oncogene Research Products) or an anti-FGF1 polyclonal antibody (23), and bands were visualized by chemiluminescence (ECL; Amersham Pharmacia Biotech). Total cell lysates were obtained from cells incubated at 37°C by resuspending cell pellets for 20 min in 0.5 ml of cold lysis buffer (20 mM Tris, pH 7.5, containing 300 mM sucrose, 60 mM KCl, 15 mM NaCl, 5% (v/v) glycerol, 2 mM EDTA, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin, and 0.2% (w/v) deoxycholate) and analyzed by immunoblot analysis.

For the analysis of the release of FGF1 and Myc-S100A13Δ88–98 from NIH 3T3 cell cotransfectants, conditioned media and cell lysates were divided into two portions, one of which was processed by heparin-Sepharose adsorption for FGF1 immunoblot analysis and the other processed by immunoprecipitation with anti-Myc antibody for the detection of Myc-S100A13Δ88–98 and Myc-S100A13. Briefly, conditioned media were filtered, treated with 0.1% (w/v) DTT for 2 h at 37°C , and concentrated using a centrifugal filter device (Ultrafree-15; Millipore Corp.) to a volume of $\sim 500\ \mu\text{l}$, and aprotinin and leupeptin were added to each sample to yield a final concentration of 2 $\mu\text{g}/\text{ml}$. Cell lysates and concentrated conditioned media were rotated for 18 h at 4°C in the presence of 1 μg of mouse anti-Myc antibody. Protein A-Sepharose (Amersham Pharmacia Biotech) was added, and the samples were rotated for an additional 2 h at 4°C . Immunoprecipitated proteins were eluted with sample buffer, boiled, resolved by 12% (w/v) SDS-PAGE, and immunoblotted with anti-Myc antibody. The activity of lactate dehydrogenase (Sigma) in conditioned media was utilized as an assessment of cell lysis in all experiments as previously reported (24).

Purification of Recombinant S100A13, Preparation of S100A13 Antiserum, and Analysis of S100A13 Heparin Affinity—The GST-S100A13 fusion protein was generated as described above (25), with the exception that the BL21 *E. coli* were lysed in 50 mM Tris buffer, pH 8.8, containing 10 mM EDTA, 10 mM glucose, and 10 $\mu\text{g}/\text{ml}$ lysozyme, and the thrombin-cleaved S100A13 protein was further purified by C8 (PerkinElmer Life Sciences) reverse-phase high pressure liquid chromatography as previously described for FGF1 (26). Female 6-month-old New Zealand White rabbits (Hazelton Research Animals) were injected intradermally with 1 mg of recombinant S100A13 suspended in Freund's complete adjuvant (Calbiochem) and boosted with 200 μg of protein suspended in Freund's incomplete adjuvant (Calbiochem). The

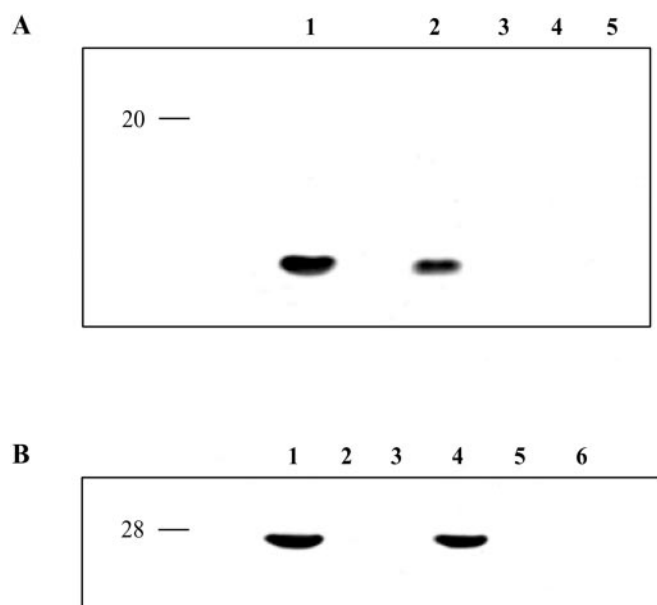


FIG. 1. The heparin affinity of S100A13. A, recombinant S100A13 was obtained as described under "Materials and Methods," and 5 μ g of recombinant S100A13 was adsorbed to heparin-Sepharose, eluted with 0.5, 1.0, and 1.5 M NaCl as described (7); the fractions were resolved by 15% (w/v) SDS-PAGE and subjected to S100A13 immunoblot analysis using a rabbit anti-S100A13 antibody. Lane 1, 5 μ g of recombinant S100A13; lane 2, 0.5 M NaCl fraction; lane 3, 1.0 M NaCl fraction; lane 4, 1.5 M NaCl fraction; lane 5, flow-through fraction. B, Myc-S100A13 NIH 3T3 cell transfectants were subjected to heat shock (110 min at 42 °C), and cell lysates were obtained from control (37 °C) and stressed cultures as described under "Materials and Methods." Proteins were adsorbed to heparin-Sepharose, eluted at 0.5, 1.0, and 1.5 M NaCl, and resolved by 15% (w/v) SDS-PAGE. Myc-S100A13 was evaluated by Myc immunoblot analysis. Lane 1, 0.5 M NaCl fraction from control cells; lane 2, 1.0 M NaCl fraction from control cells; lane 3, 1.5 M NaCl fraction from control cells; lane 4, 0.5 M NaCl fraction from heat-shocked cells; lane 5, 1.0 M NaCl fraction from heat-shocked cells; lane 6, 1.5 M NaCl fraction from heat-shocked cells.

antibody was purified by incubating the serum with immobilized recombinant S100A13 on a Problot polyvinylidene difluoride membrane (Applied Biosystem Inc.) blocked at 42 °C with 24 mM Tris, pH 7.4, containing 136 mM NaCl, 2 mM KCl, 0.1% (v/v) Tween 20, and 5% (w/v) bovine serum albumin. The membrane was washed three times with 0.05% (v/v) Triton X-100 in 50 mM Tris, pH 7.4, containing 150 mM NaCl, and the antibody was eluted with 0.2 M glycine, pH 2.8, and neutralized with 1 M K_2HPO_4 , pH 7.4. For the evaluation of S100A13 heparin affinity, 5 μ g of recombinant S100A13 were adsorbed to heparin-Sepharose (1 ml), previously equilibrated with TEB. The adsorbed proteins were washed with TEB and eluted with 2.5 ml of TEB containing increasing concentrations of NaCl. Eluted proteins were concentrated using Centricon 3 (Amicon, Inc.), resolved by 15% (w/v) SDS-PAGE, and immunoblotted with the rabbit anti-S100A13 antibody.

RESULTS

S100A13 Exhibits Low Heparin Affinity and Is Constitutively Released *In Vitro*—Because we have previously characterized ovine brain-derived S100A13 as a component of a high affinity heparin-binding multiprotein aggregate/complex containing FGF1 and the p40 extravesicular domain of p65 Syt1 (9), we sought to characterize the heparin-binding character of S100A13. As shown in Fig. 1A, while recombinant murine S100A13 was able to bind heparin-Sepharose, its affinity for heparin was low, since S100A13 immunoblot analysis reported its presence in the 0.5 M NaCl elution fraction. Thus, like p40 Syt1 (7), brain-derived S100A13 (9) may gain heparin affinity by its ability to associate either directly or indirectly with p40 Syt1 and/or FGF1.

Because the level of endogenous S100A13 expression in NIH 3T3 cells is low (data not shown), we constructed a chimera in

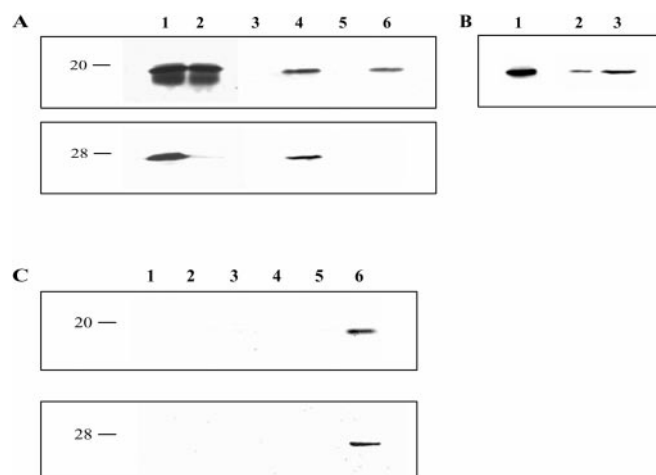


FIG. 2. The release of Myc-S100A13 in response to temperature stress. A and B, S100A13 is released with a Myc reporter in response to heat shock. Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants and insert-less vector and FGF1 NIH 3T3 cell cotransfectants (A) and Myc-S100A13 NIH 3T3 cell transfectants (B) were either subjected to heat shock (110 min at 42 °C) or incubated for 110 min at 37 °C. Conditioned media were treated with 0.1% DTT, adsorbed to heparin-Sepharose, and eluted at 1.5 M NaCl. Eluted proteins were concentrated, resolved by 15% (w/v) SDS-PAGE, and analyzed by FGF1 and Myc immunoblot analysis. A, upper panel, FGF1 immunoblot analysis; lower panel, Myc immunoblot analysis. Lane 1, total cell lysate from Myc-S100A13 and FGF1 NIH 3T3 cotransfectants; lane 2, total cell lysate from insert-less vector and FGF1 NIH 3T3 cotransfectants; lanes 3 and 4, media conditioned at 37 and 42 °C from Myc-S100A13 and FGF1 NIH 3T3 cotransfectants; lanes 5 and 6, media conditioned at 37 and 42 °C from insert-less vector and FGF1 NIH 3T3 cotransfectants. B, lane 1, total cell lysate from Myc-S100A13 NIH 3T3 transfectants; lanes 2 and 3, media conditioned at 37 and 42 °C from Myc-S100A13 NIH 3T3 transfectants. C, the kinetics of FGF1 and Myc-S100A13 release in response to temperature stress. Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants were subjected to heat shock for 10 (lane 2), 20 (lane 3), 30 (lane 4), 60 (lane 5), and 90 (lane 6) min or incubated for 90 min at 37 °C (lane 1). Conditioned media were processed as described above and analyzed by FGF1 (upper panel) and Myc (lower panel) immunoblot analysis.

which six copies of the Myc epitope were fused at the 5'-end of the S100A13 cDNA and obtained stable Myc-S100A13 NIH 3T3 cell transfectants and FGF1 and Myc-S100A13 NIH 3T3 cell cotransfectants. As a control, we also obtained FGF1 NIH 3T3 cells cotransfected with the insert-less vector used to transfect the Myc-S100A13 cDNA. Myc immunoblot analysis of cell lysates from Myc-S100A13 NIH 3T3 cell transfectants, Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants, and insert-less vector and FGF1 NIH 3T3 cell cotransfectants confirmed the expression of Myc-S100A13 as a polypeptide with an apparent molecular mass of 27 kDa in the Myc-S100A13 NIH 3T3 cell transfectants (Fig. 2B) and Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants (Fig. 2A). This band was not present in cell lysates derived from the insert-less vector and FGF1 NIH 3T3 cell cotransfectants (Fig. 2A). Myc epitope immunoblot analysis demonstrated a slight retardation in the electrophoretic mobility of Myc-S100A13 from the expected molecular mass of 21 kDa, an alteration that may be due to a change in the conformation of the protein upon binding to metal ions. Indeed, this observation is consistent with similar alterations established for a variety of other S100 proteins (27–29). As shown in Fig. 1B, the Myc-S100A13 chimera obtained from lysates of Myc-S100A13 NIH 3T3 cell transfectants exhibited similar heparin affinity as the S100A13 recombinant protein purified from the prokaryotic expression system. Because temperature stress of the Myc-S100A13 NIH 3T3 cell transfectants did not alter the heparin affinity of the Myc-S100A13 chimera (Fig. 1B), we suggest that the Myc-S100A13 protein may not be

subjected to temperature-mediated post-translational modifications, which may affect its heparin affinity.

We have previously reported that NIH 3T3 cells transfected with FGF1 and p65 Syt1 are able to release FGF1 and the p40 extravesicular domain of Syt1 in response to cellular stress (7, 8) and that FGF1 and the p40 extravesicular domain of p65 Syt1 are present in the extracellular compartment as a reducing agent- and denaturant-sensitive complex (7). Because the expression of p40 Syt1 in NIH 3T3 cells results in the stress-independent, constitutive release of p40 Syt1 (8), we questioned whether Myc-S100A13 may also be able to access the extracellular compartment either spontaneously or under conditions of temperature stress. Myc-S100A13 NIH 3T3 cell transfectants were subjected to heat shock (110 min at 42 °C) or maintained for 110 min at 37 °C, and conditioned media were treated with 0.1% (w/v) DTT, adsorbed to heparin-Sepharose, eluted at 1.5 M NaCl, and analyzed for Myc-S100A13 release by immunoblot analysis. As shown in Fig. 2B, we observed that Myc-S100A13 was present in media conditioned independently of temperature stress and that stress conditions did not significantly increase the level of Myc-S100A13 in the extracellular compartment. Kinetic analysis of the temperature-independent Myc-S100A13 release from Myc-S100A13 NIH 3T3 cell transfectants demonstrated that Myc-S100A13 was detectable in media conditioned at 37 °C after 30 min with an increase in the level of extracellular Myc-S100A13 observed after prolonged time periods (hours) at 37 °C (data not shown).

FGF1 Is Able to Repress the Constitutive Release of S100A13—The Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants were also subjected to heat shock in order to study the simultaneous release of Myc-S100A13 and FGF1. Conditioned media were obtained from both Myc-S100A13 and FGF1 and insert-less vector and FGF1 NIH 3T3 cell cotransfectants following heat shock and processed for FGF1 and Myc immunoblot analysis. We observed that both cotransfectants released similar levels of FGF1 in response to stress, and the Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants also released the Myc-S100A13 chimera in response to temperature stress (Fig. 2A). The Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants were further evaluated for the capacity to constitutively release Myc-S100A13 independent of stress, and Myc immunoblot analysis was able to detect the presence of Myc-S100A13 only in media conditioned at 37 °C for time periods greater than 8 h (data not shown). Further, FGF1 immunoblot analysis revealed that FGF1 was not present in media conditioned at 37 °C for this prolonged time period (data not shown). These data suggest that (i) the constitutive release of Myc-S100A13 at 37 °C is significantly impaired but is up-regulated in response to stress in a FGF1-rich background represented by the Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants and (ii) the constitutive release of Myc-S100A13 occurs unattenuated in a FGF1-poor background represented by the Myc-S100A13 NIH 3T3 cell transfectants.

Because FGF1 and p40 Syt1 are known to be released in response to heat shock with rather slow kinetics (8), we evaluated whether the stress-induced release of Myc-S100A13 also proceeds with similar kinetics. The Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants were subjected to heat shock for 10, 20, 30, 60, and 90 min, and the conditioned media were processed for FGF1 and Myc immunoblot analysis. As shown in Fig. 2C, both FGF1 and Myc-S100A13 were released with similar kinetics, and both required at least 90 min of exposure to temperature stress to gain access to the extracellular compartment. These results suggest that FGF1 (6), p40 Syt1 (8), and Myc-S100A13 also exhibit similar kinetics of release from NIH 3T3 cells in response to stress when NIH 3T3 cell Myc-S100A13

transfectants are cotransfected with FGF1.

The Expression of Myc-S100A13 Overcomes the Transcriptional and Translational Requirements for FGF1 Release in Response to Heat Shock—Since we have previously reported that the release of FGF1 and p40 Syt1 is dependent on transcription and translation (3, 8), we evaluated the effect of actinomycin D and cycloheximide on the heat shock-induced release of Myc-S100A13 and FGF1. Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants and insert-less vector and FGF1 NIH 3T3 cell cotransfectants were subjected to heat shock in the presence and absence of actinomycin D (10 µg/ml) or cycloheximide (10 µg/ml), and conditioned media were analyzed for FGF1 and Myc-S100A13 release by immunoblot analysis. We observed that FGF1 NIH 3T3 cells cotransfected with Myc-S100A13 were able to release FGF1 and Myc-S100A13 in response to heat shock in the presence of either actinomycin D or cycloheximide (Fig. 3B). In contrast, the treatment of insert-less vector and FGF1 NIH 3T3 cell cotransfectants with actinomycin D and cycloheximide completely inhibited FGF1 release (Fig. 3A). Because these data demonstrated that Myc-S100A13 expression was able to revert the sensitivity of FGF1 release to actinomycin D and cycloheximide, we evaluated whether S100A13 gene expression was induced during heat shock. However, analysis of the steady-state levels of the transcripts for S100A13 at 37 and 42 °C demonstrated that S100A13 is not a heat shock gene (data not shown). Thus, we suggest that the ability of Myc-S100A13 expression to revert the actinomycin D- and cycloheximide-sensitive component of the FGF1 release pathway may be due to a heat shock-induced post-translational event mediated by S100A13.

Because we have previously reported that the stress-mediated release of FGF1 and p40 Syt1 requires the biosynthesis of ATP (8), is sensitive to reagents that affect the integrity of the actin cytoskeleton (9, 16), and is not affected by brefeldin A (8), a reagent that disrupts communication between the ER-Golgi apparatus (30), we sought to determine whether the stress-induced release of Myc-S100A13 exhibited similar behavior. Thus, Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants were subjected to heat shock in the presence and absence of the appropriate biochemical reagent, and their conditioned media were subjected to Myc and FGF1 immunoblot analysis. As shown in Fig. 3C, the release of FGF1 and Myc-S100A13 was inhibited by treatment with (i) 2-deoxyglucose, a reagent that inhibits ATP biosynthesis (31), and (ii) amlexanox and latrunculin, reagents that induce the collapse of actin cytoskeleton through different mechanisms (16, 32). In addition, the release of Myc-S100A13 and FGF1 was insensitive to treatment with brefeldin A, a reagent that disrupts ER-Golgi communication (30).

Since Myc-S100A13 is constitutively released from Myc-S100A13 NIH 3T3 cell transfectants and its release is significantly inhibited by the expression of FGF1, we evaluated the pharmacological properties of the constitutive release of S100A13. We observed that, like the constitutive release of p40 Syt1 (8), the constitutive release of Myc-S100A13 is insensitive to amlexanox, 2-deoxyglucose, brefeldin A, actinomycin D, and cycloheximide (data not shown). In contrast, amlexanox and 2-deoxyglucose are able to repress the release of both FGF1 and Myc-S100A13 in response to heat shock from FGF1 and Myc-S100A13 NIH 3T3 cell cotransfectants. Furthermore, while FGF1 is able to repress the release of S100A13, it is not able to repress the constitutive release of p40 Syt1 (8). These data suggest that the constitutive release of both Myc-S100A13 and p40 Syt1 occurs using pathways divergent from the stress-dependent pathway of FGF1 release and also argues that the constitutive release pathways exhibited by Myc-S100A13 and p40 Syt1 (8) may be different.

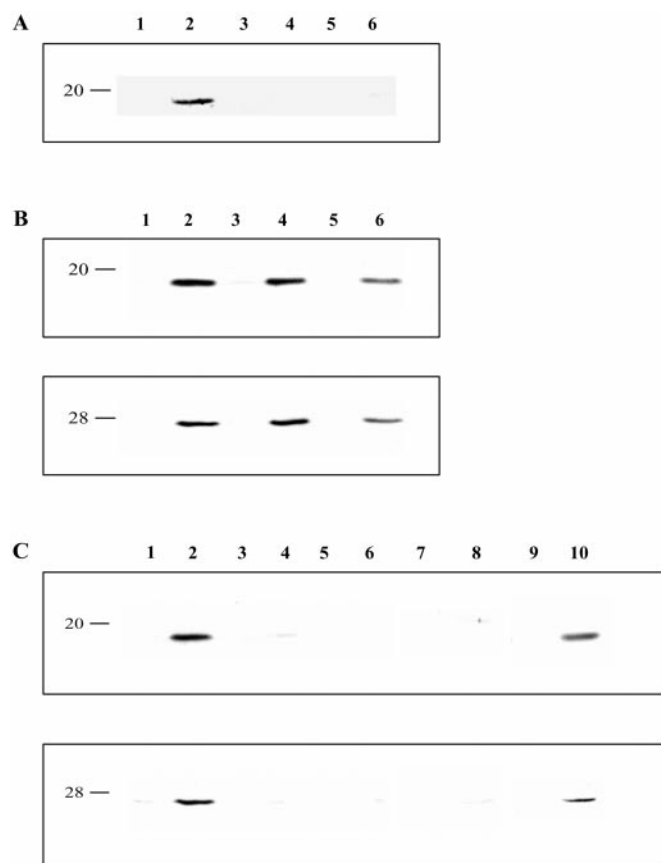


FIG. 3. Pharmacology of FGF1 and Myc-S100A13 release in response to temperature stress. A and B, the ability of Myc-S100A13 to overcome the requirement for transcription and translation for FGF1 release in response to temperature stress. Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants (B) and insert-less vector and FGF1 NIH 3T3 cell cotransfectants (A) were incubated for 2 h at 37 °C in the presence and absence of 10 μ M cycloheximide or 10 μ M actinomycin D and then either subjected to heat shock (110 min at 42 °C) or incubated for 110 min at 37 °C in the presence and absence of the same concentration of the two reagents. Conditioned media were treated with 0.1% (w/v) DTT, adsorbed to heparin-Sepharose, and eluted at 1.5 M NaCl. Eluted proteins were concentrated, resolved by 15% (w/v) SDS-PAGE, and analyzed by FGF1 and Myc immunoblot analysis. A, FGF1 immunoblot analysis. B, upper panel, FGF1 immunoblot analysis; lower panel, Myc immunoblot analysis. Lanes 1 and 2, media conditioned at 37 and 42 °C from control cells; lanes 3 and 4, media conditioned at 37 and 42 °C from cells incubated with cycloheximide; lanes 5 and 6, media conditioned at 37 and 42 °C from cells incubated with actinomycin D. C, effect of amlexanox, latrunculin, 2-deoxyglucose, and brefeldin A on FGF1 and Myc-S100A13 release in response to temperature stress. Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants were subjected to heat shock in the presence and absence of 0.375 mM amlexanox, 400 nM latrunculin, 50 mM 2-deoxyglucose, and 0.5 μ M brefeldin A. For the evaluation of brefeldin A and 2-deoxyglucose activity, the cells were pretreated prior to the heat shock with the same concentrations of the reagents for 30 and 60 min, respectively, and conditioned media were collected and processed as described above. Upper panel, FGF1 immunoblot analysis; lower panel, Myc immunoblot analysis: lanes 1 and 2, media conditioned at 37 and 42 °C from control cells; lanes 3 and 4, media conditioned at 37 and 42 °C from amlexanox-treated cells; lanes 5 and 6, media conditioned at 37 and 42 °C from latrunculin-treated cells; lanes 7 and 8, media conditioned at 37 and 42 °C from 2-deoxyglucose-treated cells; lanes 9 and 10, media conditioned at 37 and 42 °C from brefeldin A-treated cells. The figure is a composite of several experiments.

Myc-S100A13 May Be Released as a Complex with FGF1 in Response to Heat Shock—The term S100 refers to the ability of S100 gene translation products to be resistant to precipitation at 100% (w/v) ammonium sulfate saturation (18). In contrast, FGF1 is precipitated by ammonium sulfate fractionation at 85% (w/v) saturation (33), and ammonium sulfate fractionation

has been utilized to fractionate FGF1 from numerous organs and tissues (9, 34). To evaluate the ability of S100A13 to be precipitated by ammonium sulfate fractionation, we performed 100% (w/v) ammonium sulfate fractionation using media conditioned by heat shock from Myc-S100A13 NIH 3T3 cell transfectants. Following dialysis, pellets and supernatants from the 100% (w/v) ammonium sulfate fractionated media were adsorbed to heparin-Sepharose and eluted with 1.5 M NaCl. As anticipated, Myc immunoblot analysis revealed that Myc-S100A13 released by the Myc-S100A13 NIH 3T3 cell transfectants in response to heat shock was present only in the supernatant fraction (data not shown).

Because these data suggested that Myc-S100A13 may be resistant to precipitation following 100% (w/v) ammonium sulfate fractionation, we hypothesized that if Myc-S100A13 is complexed with FGF1 as a result of stress-induced release, the Myc-S100A13- and FGF1-containing complex should be characterized by enhanced solubility of FGF1 in the supernatant fraction following 100% (w/v) ammonium sulfate saturation. To assess this premise, media conditioned by heat shock from Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants and from insert-less vector and FGF1 NIH 3T3 cell cotransfectants were subjected to 100% (w/v) ammonium sulfate fractionation and pellet and supernatant fractions obtained by centrifugation. After DTT treatment, pellet and supernatant fractions were adsorbed to heparin-Sepharose, eluted at 1.5 M NaCl, and the presence of FGF1 and Myc-S100A13 assessed by FGF1 and Myc immunoblot analysis. As shown in Fig. 4A, higher levels of FGF1 were resolved in the pellet fraction from media obtained from temperature-stressed insert-less vector and FGF1 NIH 3T3 cell cotransfectants, whereas media obtained from temperature-stressed Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants exhibited increased levels of FGF1 and Myc-S100A13 in the supernatant fraction. These data suggest that Myc-S100A13 may be able to alter the solubility of FGF1 in media conditioned by heat shock, and the solubility of FGF1 under conditions of 100% (w/v) ammonium sulfate saturation may be a consequence of an association between FGF1 and Myc-S100A13.

A S100A13 Mutant Lacking the Basic Residue-rich Domain Functions as a Dominant Negative Regulator of FGF1 Release—The carboxyl-terminal domains of several S100 proteins have been described as the structural regions responsible for mediating the interaction between S100 polypeptides and specific target proteins (17, 18). Because the carboxyl terminus of S100A13 (last 11 amino acid residues) is characterized by the presence of 6 basic amino acid residues (Fig. 5A), a feature absent in the other members the S100 protein family (19), we evaluated whether the carboxyl-terminal basic residue-rich domain present in S100A13 is involved in the release of FGF1 and Myc-S100A13. We produced a deletion mutant of Myc-S100A13 (Myc-S100A13 Δ 88–98) lacking the last 11 residues at the carboxyl terminus of the protein and cotransfected FGF1 NIH 3T3 cell transfectants with this deletion mutant. Analysis of the heparin affinity of the Myc-S100A13 Δ 88–98 mutant obtained from cell lysates of the FGF1 and Myc-S100A13 Δ 88–98 NIH 3T3 cell cotransfectants demonstrated that the Myc-S100A13 Δ 88–98 protein is devoid of heparin affinity (data not shown), suggesting that the basic residue-rich region of Myc-S100A13 may be responsible for its ability to associate with heparin.

We also evaluated the ability of the FGF1 and Myc-S100A13 Δ 88–98 NIH 3T3 cell cotransfectants to release FGF1 and the Myc-S100A13 Δ 88–98 deletion mutant in response to temperature stress. The FGF1 and Myc-S100A13 NIH 3T3 cell cotransfectants were used as a positive control. The cotransfec-

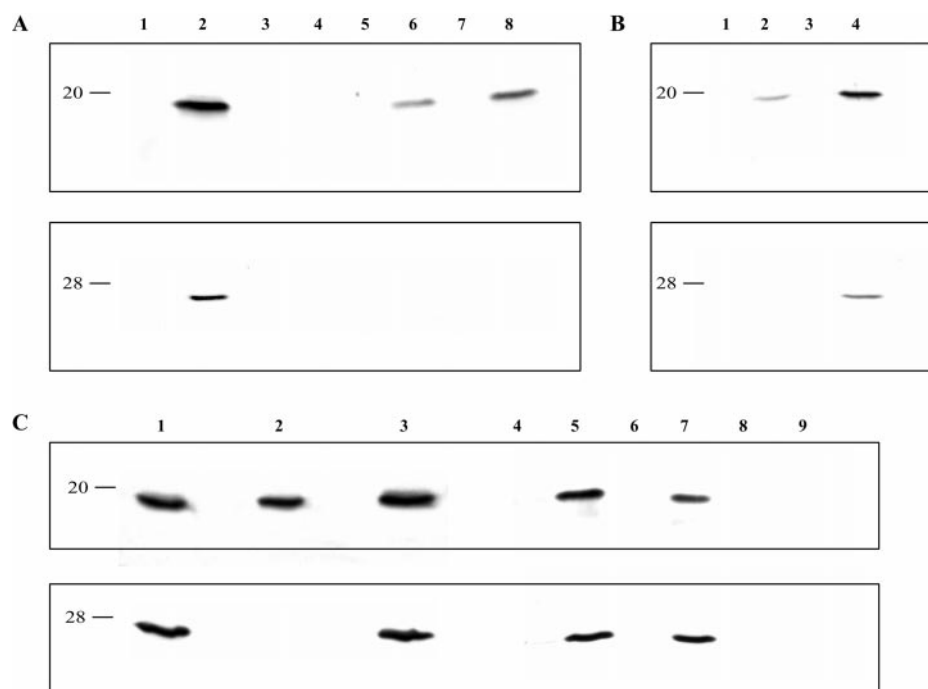


FIG. 4. Ammonium sulfate fractionation and the release of FGF1 and Myc-S100A13 in response to heat shock. A, solubility of Myc-S100A13 and FGF1 at ammonium sulfate saturation. Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants and insertless vector and FGF1 NIH 3T3 cell cotransfectants were subjected to heat shock; conditioned media were collected and subjected to 100% (w/v) ammonium sulfate fractionation; and pellet and supernatant fractions were obtained by centrifugation. After DTT treatment, the pellet and supernatant fractions were adsorbed to heparin-Sepharose, eluted at 1.5 M NaCl, resolved by 15% (w/v) SDS-PAGE, and evaluated by FGF1 and Myc immunoblot analysis. *Upper panel*, FGF1 immunoblot analysis; *lower panel*, Myc immunoblot analysis. *Lanes 1 and 2*, 37 and 42 °C supernatant fractions from Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants; *lanes 3 and 4*, 37 and 42 °C pellet fractions from Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants; *lanes 5 and 6*, 37 and 42 °C supernatant fractions from insert-less vector and FGF1 NIH 3T3 cell cotransfectants; *lanes 7 and 8*, 37 and 42 °C pellet fractions from insert-less vector and FGF1 NIH 3T3 cell cotransfectants. B, solubility of Myc-S100A13 and Cys-free FGF1 at ammonium sulfate saturation. Myc-S100A13 and Cys-free FGF1 NIH 3T3 cell cotransfectants were subjected to heat shock; conditioned media were collected and subjected to 100% (w/v) ammonium sulfate fractionation; and pellet and supernatant fractions were obtained as described above and analyzed by FGF1 and Myc immunoblot analysis. *Upper panel*, FGF1 immunoblot analysis; *lower panel*, Myc immunoblot analysis. *Lanes 1 and 2*, 37 and 42 °C pellet fractions from Myc-S100A13 and Cys-free FGF1 NIH 3T3 cell cotransfectants; *lanes 3 and 4*, 37 and 42 °C supernatant fractions from Myc-S100A13 and Cys-free FGF1 NIH 3T3 cell cotransfectants. C, ability of Myc-S100A13 to export Cys-free FGF1 in response to temperature stress. Myc-S100A13 and FGF1, Myc-S100A13 and Cys-free FGF1, and insertless vector and Cys-free FGF1 NIH 3T3 cell cotransfectants were subjected to heat shock, and conditioned media were collected, treated with 0.1% (w/v) DTT, adsorbed to heparin-Sepharose, and eluted with 1.5 M NaCl. The eluted fractions were resolved by 15% (w/v) SDS-PAGE and analyzed by FGF1 and Myc immunoblot analysis. *Upper panel*, FGF1 immunoblot analysis; *lower panel*, Myc immunoblot analysis. *Lane 1*, total cell lysate from Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants; *lane 2*, total cell lysate from insert-less vector and Cys-free FGF1 NIH 3T3 cell cotransfectants; *lane 3*, total cell lysate from Myc-S100A13 and Cys-free FGF1 NIH 3T3 cell cotransfectants; *lanes 4 and 5*, media conditioned at 37 and 42 °C from Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants; *lanes 6 and 7*, media conditioned at 37 and 42 °C from Myc-S100A13 and Cys-free FGF1 NIH 3T3 cell cotransfectants; *lanes 8 and 9*, media conditioned at 37 and 42 °C from insert-less vector and Cys-free FGF1 NIH 3T3 cell cotransfectants.

tants were subjected to heat shock and, following DTT treatment, the conditioned media were either adsorbed to heparin-Sepharose for the evaluation of FGF1 release or concentrated and immunoprecipitated with an anti-Myc antibody for an evaluation of Myc-S100A13 Δ 88–98 release. FGF1 immunoblot analysis demonstrated that while FGF1 was present in media conditioned by temperature stress from FGF1 and Myc-S100A13 NIH 3T3 cell cotransfectants, FGF1 was not detected in media conditioned by heat shock from FGF1 and Myc-S100A13 Δ 88–98 NIH 3T3 cell cotransfectants (Fig. 5B). In contrast, however, Myc-S100A13 Δ 88–98 was present in media conditioned by heat shock from the FGF1 and Myc-S100A13 Δ 88–98 NIH 3T3 cell cotransfectants (Fig. 5C). These data suggest that the basic residue-rich domain in Myc-S100A13 may not be required for the heat shock-induced export of Myc-S100A13, but this domain may be necessary for the export of FGF1 into the extracellular compartment in response to temperature stress.

The Expression of Myc-S100A13 Induces the Release of Cys-free FGF1 in Response to Temperature Stress—We have previously reported that (i) FGF1 is released as a homodimer in response to heat shock (3, 6), (ii) a Cys-free mutant of FGF1 is not able to access the extracellular compartment in response to

stress (6), and (iii) residue Cys³⁰ in FGF1 is responsible for dimer formation and export into the extracellular compartment (35). Since the solubility of extracellular FGF1 under conditions of 100% (w/v) ammonium sulfate saturation suggests that S100A13 and FGF1 may be able to associate, we evaluated whether S100A13 was able to affect the release of the Cys-free mutant of FGF1. As a result, the Cys-free FGF1 NIH 3T3 cell transfectants were cotransfected with either Myc-S100A13 or insertless vector, and media conditioned by temperature stress from both cotransfectants were evaluated for the presence of FGF1 and Myc-S100A13. FGF1 and Myc immunoblot analysis revealed that while the Cys-free FGF1 mutant was not released in response to heat shock from the insertless vector control and FGF1 cotransfectants, the Cys-free FGF1 mutant was released in response to temperature stress in cells expressing both Cys-free FGF1 and the Myc-S100A13 chimera (Fig. 4C). In addition, the Myc-S100A13 and Cys-free FGF1 NIH 3T3 cell cotransfectants were also able to release Myc-S100A13 in response to heat shock (Fig. 4C). Moreover, as previously described for the Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants, Cys-free FGF1 was also able to suppress the constitutive release of Myc-S100A13 (Fig. 4C). The heat shock-induced release of Cys-free FGF1 and Myc-S100A13 from Cys-

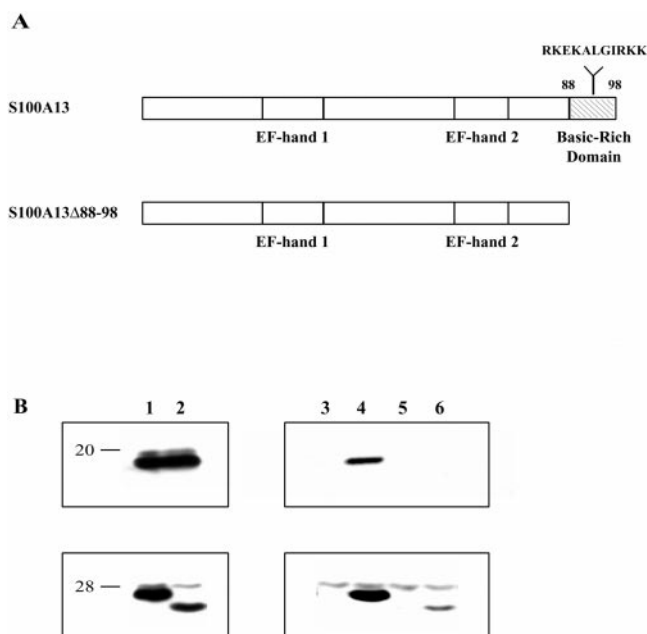


FIG. 5. Ability of Myc-S100A13Δ88-98 to inhibit the release of FGF1 in response to temperature stress. A, schematic illustration of the domain structure of S100A13 (*top*) and the deletion mutant S100A13Δ88-98 (*bottom*). B, Myc-S100A13 and FGF1 and S100A13Δ88-98 and FGF1 NIH 3T3 cell cotransfectants were subjected to heat shock, and, following DTT treatment, conditioned media were either adsorbed to heparin-Sepharose for evaluation of FGF1 release or concentrated and immunoprecipitated with anti-Myc antibody for the evaluation of Myc-S100A13Δ88-98 and Myc-S100A13 release. Eluted and immunoprecipitated proteins were resolved by 15% (w/v) and 12% (w/v) SDS-PAGE, respectively, and evaluated by FGF1 and Myc immunoblot analysis. *Upper panel*, FGF1 immunoblot analysis; *lower panel*, Myc immunoblot analysis. *Lane 1*, cell lysate from Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants; *lane 2*, cell lysate from Myc-S100A13Δ88-98 and FGF1 NIH 3T3 cell cotransfectants; *lanes 3 and 4*, media conditioned at 37 and 42 °C from Myc-S100A13 and FGF1 NIH 3T3 cell cotransfectants; *lanes 5 and 6*, media conditioned at 37 and 42 °C from Myc-S100A13Δ88-98 and FGF1 NIH 3T3 cell cotransfectants.

free FGF1 and Myc-S100A13 NIH 3T3 cell cotransfectants (i) occurred with kinetics similar to that observed for the release of FGF1, (ii) was sensitive to amlexanox and 2-deoxyglucose treatment, two reagents that are able to inhibit the stress-induced release of FGF1 (6, 7), (iii) was insensitive to brefeldin A, a feature also exhibited by the release of FGF1, and (iv) was insensitive to treatment with actinomycin D and cycloheximide (data not shown). These data suggest that the expression of Myc-S100A13 may be able to affect the release of FGF1 either as a monodimer or as a noncovalent dimer.

Since our results have suggested that Myc-S100A13 is able to (i) associate with FGF1 in response to temperature stress and (ii) induce the temperature-dependent release of Cys-free FGF1, we evaluated the capacity of Myc-S100A13 to be released in association with Cys-free FGF1. Myc-S100A13 and Cys-free FGF1 NIH 3T3 cell cotransfectants were subjected to heat shock, conditioned media were collected and fractionated at 100% (w/v) ammonium sulfate, and pellet and supernatant fractions were obtained. As shown in Fig. 4B, FGF1 and Myc immunoblot analysis revealed the presence of significant levels of Cys-free FGF1 and Myc-S100A13 in the supernatant fraction, suggesting that Myc-S100A13 may also be able to associate with the monomeric form of FGF1 under conditions of temperature stress.

DISCUSSION

Our data suggest that S100A13 is involved in the regulation of the stress-induced FGF1 release pathways. Thus, like the

stress-induced release of FGF1 (3), p40 Syt1 (7, 8), and murine IL-1α (14), the stress-induced release of Myc-S100A13 is sensitive to agents that interfere with ATP biosynthesis and organization of the F-actin cytoskeleton but is insensitive to disruption of intracellular communication between the ER-Golgi apparatus. However, several observations suggest that S100A13, unlike p40 Syt1, is able to facilitate the release of FGF1. Indeed, expression of Myc-S100A13 was able to overcome the inhibitory activity of actinomycin and cycloheximide on FGF1 release and was able to induce the release of Cys-free FGF1 in response to stress, suggesting a functional role for S100A13 in the FGF1 release pathway as a potential modifier of a stress-induced post-translational event.

The finding that Myc-S100A13 expression is able to overcome the requirement for transcription and translation in the release of FGF1 is consistent with the observation that some members of the S100 gene family have been characterized not only as stress-induced genes (36–39) but also as Cu²⁺-binding proteins (17, 18, 40, 41). It is interesting to note that S100B was independently characterized from neural tissue as an inhibitor of Cu²⁺-mediated L-ascorbate oxidation (40). Moreover, S100A4, S100A6, S100A7, and S100B are up-regulated in several human tumor cells, where they are associated with increased invasiveness of transformed cells and acquisition of metastatic phenotype (17). The murine analog of S100A8, the CP-10 protein, has been also associated with inflammation, since it is expressed and released in macrophages and endothelial cells only after their activation by interleukin-1 and lipopolysaccharide (17, 42). However, the observation that the S100A13 gene is not induced in response to heat shock in NIH 3T3 cells does not eliminate the possibility that other members of the S100 gene family may participate in the release of FGF1 and that the expression of S100A13 may compensate for their function.

While it is difficult to anticipate how S100A13 participates in these release pathways, we suggest that S100A13 may be able to orient FGF1 and Cys-free FGF1 in a manner that enables these polypeptides to form noncovalent homodimers. Indeed, Cu²⁺ oxidation is able to induce FGF1 homodimer (26) and FGF1 IL-1α heterodimer formation (26). Additional evidence from S100 crystallographic studies demonstrates that members of the S100 gene family are able to form stable Ca²⁺-independent homodimers and through an alteration in their conformation enable the two EF-hands in S100 to hold polypeptides, such as annexin 2 (Anx2) (43), enabling the carboxyl-terminal domain of the polypeptide to interact with other proteins (17, 18). In studies utilizing the interaction between S100A11 and Anx1 (44, 45) as well as between S100A10 and Anx2 (43), the orientation established by these conformational changes yields the formation of stable heterotetramers (S100A11₂Anx1₂ and S100A10₂Anx2₂). In the situations with FGF1, it is intriguing to speculate that S100A13 may be able to form similar heterotetramers with these polypeptides. Indeed, this may explain how S100A13 is able to facilitate the release of Cys-free FGF1, since it is possible that when interacting with native FGF1, S100A13 may be able to orient its conformation to expose Cys³⁰, which is not exposed to solvent in its native conformation (46), for FGF1 homodimer oxidation. In the absence of Cys³⁰, S100A13 may enable Cys-free FGF1 to form the noncovalent equivalent of the FGF1 Cys³⁰ homodimer as a component of a FGF₂:S100A13₂ heterotetramer so that it can be released in response to heat shock. This would be consistent with the observation that the stress-induced release of Cys-free FGF1 and Myc-S100A13 exhibits similar kinetics and responsiveness to pharmacological agents including amlexanox, 2-deoxyglucose, brefeldin A, cycloheximide, and actino-

mycin D (data not shown). In addition, the S100A13-dependent increase in the solubility of Cys-free FGF1 under conditions of 100% (w/v) ammonium sulfate saturation also supports the premise that S100A13 may be able to associate with the monomeric form of FGF1 in response to temperature stress, and this association may involve the formation of a noncovalent heterotetramer complex that may enable Cys-free FGF1 to access the release pathway. Interestingly, the ability of S100A13 to force FGF1 into the supernatant fraction under conditions of saturated ammonium sulfate fractionation would be consistent with this suggestion, since solubility in ammonium sulfate is well described as being sensitive to alterations in protein conformation (47).

It is interesting that latrunculin and amlexanox, which are known to attenuate the F-actin cytoskeleton (16, 32), also inhibit the heat shock-induced release of FGF1 and Myc-S100A13. Indeed, this suggests that the cytoskeleton may play an important role in the FGF1 release pathway. The premise is further supported by the association of the members of the S100 gene family with actin stress fibers, which is dependent upon the physiological status of the cell (48). As a result, it is possible that S100A13 may act as an adaptor between FGF1 and F-actin structures. The F-actin cytoskeleton may be involved in at least two stages of the stress-induced release of FGF1 and Myc-S100A13: (i) the transport of the complex to the cell membrane proceeding along F-actin stress fibers with the potential participation of myosin molecular motors (49) and (ii) exocytosis, which has been demonstrated to depend on the submembrane actin cortex during classical protein secretion (50). Because both actin-dependent transport and exocytosis are energy-dependent processes (50), this suggestion is consistent with the ability of 2-deoxyglucose to repress the stress-induced release of FGF1 and Myc-S100A13. Interestingly, unlike latrunculin (32), amlexanox does not interfere with the stability of submembrane F-actin (16), and this further implies that the F-actin stress fibers may be involved in the regulation of the transport of FGF1, p40 Syt1, and Myc-S100A13 in the nonclassical release of these polypeptides.

The observation that S100A13 is constitutively released *in vitro* is also noteworthy, since a similar observation has been made with the intracellular p40 fragment of p65 Syt1 (8). Interestingly, the function of the C2A domain in p40 Syt1 has been implicated in lipid bilayer penetration (51). However, unlike p40 Syt1, which exhibits constitutive release in FGF1 NIH 3T3 cell transfectants (8), the expression of FGF1 in Myc-S100A13 NIH 3T3 cell transfectants represses the constitutive release of Myc-S100A13. Although it is difficult to interpret the significance of these data, it is possible that the ability of FGF1 to attenuate constitutive release of Myc-S100A13 but not the constitutive release of p40 Syt1 may reflect the ability of intracellular FGF1 to prefer an association with S100A13 rather than with p40 Syt1. Indeed, the observation that FGF1, S100A13, and p40 Syt1 are present as a noncovalent aggregate/complex in neural tissue suggests that the self-aggregation properties attributed to both the extravesicular p40 domain of p65 Syt1 (52) and S100 gene family members (17, 18) may be involved in the arrangement of a conformation-sensitive aggregate complex that may facilitate the release of FGF1. The observations that FGF1 (35), p40 Syt1 (53), and S100A13 (54) are able to associate with acidic phospholipids further suggest an additional complexity to the stoichiometric interactions between these polypeptides.

Although we do not know how this multiprotein aggregate/complex gains access to the extracellular compartment, it is likely that the basic residue-rich domain at the carboxyl terminus of S100A13 may be involved in regulating this function.

Several studies have reported that the carboxyl-terminal domain of S100 proteins is involved in mediating the interaction of S100 polypeptides in their dimeric state with their target proteins (17, 18). Because deletion of the basic residue-rich domain at the carboxyl terminus of Myc-S100A13 results in the generation of a dominant-negative effector of FGF1 release in response to temperature stress, it is likely that this domain may be involved in the regulation of FGF1 export under heat shock conditions. However, it is unlikely that the S100A13 basic residue-rich domain is involved in mediating the ability of Myc-S100A13 to traverse the plasma membrane, since the Myc-S100A13 Δ 88–98 mutant is released in response to heat shock. This is consistent with the observations that members of the S100 gene family lacking a basic residue-rich domain at their carboxyl terminus are released into the extracellular compartment following expression in mammalian cells (17, 18, 21) and may imply that the carboxyl-terminal basic residue-rich domain in S100A13 functions to associate with target proteins, while the remainder of the S100A13 may be involved in penetration through the lipid bilayer. Indeed, S100B and S100A10 do not contain a basic residue-rich domain, yet S100B is released by glial cells (55, 56), and S100A10 has been reported not only to associate with plasminogen in the extracellular compartment but is able to stimulate tissue-dependent plasminogen activation either alone or as a complex with Anx2 (57). These and other studies (58, 59) have implicated the function of S100, Anx2, and phosphatidylserine as key mediators of the extrinsic coagulation and fibrinolytic systems on the surface of the endothelial cell (57–59). Further, phosphatidylserine flipping from the inner leaflet to the outer leaflet of the plasma membrane is known to be a regulator of vascular hemostasis (60), and phosphatidylserine is also able to flip to the outer leaflet of the plasma membrane in response to heat shock (60). Because all of the known components of the stress-induced FGF1 release pathway have been characterized as phosphatidylserine-binding proteins (35, 53, 54) and S100A13 has been characterized as an Anx2-binding protein (61), we anticipate that Anx2 and phosphatidylserine flipping may be involved in mediating the export of these polypeptides.

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